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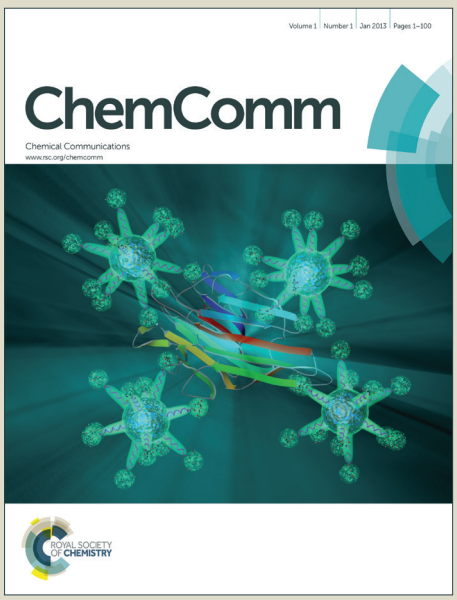

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DOI: 10.1039/C4CC07320G

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Out of the active site binding pocket for Carbonic Anhydrase inhibitors†

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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A structural study of the adduct which 2-benzylsulfinylbenzoic acid forms with human carbonic anhydrase II is reported, showing a binding mode completely different from any other class of carbonic anhydrase inhibitors investigated so far: this carboxylate binds in a pocket situated out of the enzyme active site.

Human carbonic anhydrases (hCAs) are zinc-requiring metalloenzymes, which catalyze the reversible hydration of carbon dioxide to bicarbonate and proton.[1,2] 15 different isoforms have been so far identified, among which 12 are catalytically active (CAs I–IV, VA–VB, VI–VII, IX, and XII–XIV), whereas the CA-related proteins (CARPs) VIII, X, and XI are devoid of catalytic activity.[1,2]
The hCAs catalytic mechanism is a well understood process, which takes place in two separate steps described by equations (1) and (2).[3,4]

$$EZn^{2+}\text{-}OH^- + CO_2 \leftrightarrows EZn^{2+}\text{-}HCO_3^- \leftrightarrows EZn^{2+}\text{-}H_2O + HCO_3^- \quad (1)$$

$$EZn^{2+}\text{-}H_2O + B \leftrightarrows EZn^{2+}\text{-}OH^- + BH^+ \quad (2)$$

The first catalytic step is the nucleophilic attack of a zinc-bound hydroxide ion on a carbon dioxide molecule entrapped within a hydrophobic pocket of the active site, which leads to the formation of a bicarbonate ion coordinated to the metal ion. The binding of bicarbonate to zinc is rather weak and thus this ion is readily substituted by a water molecule, generating the acidic form of CA, which is catalytically unproductive (equation 1). In the second step of the reaction, which is the rate limiting one, the zinc-bound hydroxide is regenerated through a proton transfer reaction from the zinc-coordinated water molecule to the external buffer.[4] This step is generally facilitated by a residue placed in the middle of the active site, which is able to assist the proton transfer reaction, acting thus as a proton shuttle. In the majority of human CA isoforms this residue is a histidine (e.g., His64 in hCA II, IV, VII, IX, etc).[5,6] In some other isoforms, the absence of this histidine has been related to the low catalytic efficiency of the enzyme. For example in hCA III, which has the lowest catalytic efficiency among the active isoforms, this histidine is substituted by a lysine.[7] Further support to the role of His64 as proton shuttle comes from the observation that in a large number of studies, conducted mainly on the ubiquitous isoform hCA II, this residue is observed in two different conformations, termed *in* and *out*.[8-10] In the *in* conformation the histidine is orientated toward the interior of the active site, probably to accept a proton from a relay of water molecules, which connect it to the zinc-bound water molecule, whereas in the *out* conformation this residue is orientated toward the external of the active site to deliver the proton to the bulk solvent.[8-10]
Although being a very simple reaction, the hydration of carbon dioxide is of fundamental importance to many processes based on gas exchange, ion transport, and pH balance, thus connecting hCAs to a variety of physiological processes. Since abnormal levels and/or activities of these enzymes have been associated with several human diseases, such as glaucoma, obesity, cancer, epilepsy, etc,[2] they represent interesting targets for pharmaceutical research. Indeed, several CA inhibitors (CAIs) are currently clinically used for the treatment or prevention of diseases such as glaucoma and epilepsy.[1,2]
The main drawback associated with the use of CAIs as drugs is related to their lack of selectivity caused by the high sequence and structural similarity of the different hCA isoforms. Thus, the last years saw a large number of efforts devoted to the development of isozyme-selective inhibitors.
At least two major classes of CAIs have been so far characterized: compounds which bind to the enzyme active site coordinating the catalytic zinc ion,[1,2] and compounds which bind in the active site but do not interact with the metal ion.[11-16] Among the zinc binders the most important CAIs are the primary sulfonamides and their isosteres, such as sulfamates and sulfamides.[1,17,18] They bind as deprotonated anions to the Zn(II) from the CA active site in tetrahedral geometries. Other zinc-binders are the dithiocarbamates,[19] xanthates[20] as well as small inorganic anions such as halides, pseudohalides, etc.[21] The second class of inhibitors is characterized by compounds that can either be anchored to the zinc-coordinated water molecule/hydroxide ion, as observed for phenols,[22,23] and polyamines,[12] or bind to the border of the active cavity partially occluding its entrance, as reported for coumarins[14,15] and lacosamide.[24]
Interestingly the carboxylic acids, a group of compounds containing many milli- micro-molar CAIs,[21] can show binding modes belonging to both classes mentioned above. Indeed, some of them (formate and acetate), coordinate to the Zn(II) ion[25-28] while others, such as phenolic acids **1** and **2** (Figure 1),[29] interact with the enzyme hydrogen bonding the zinc-bound water molecule. However the

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molecular determinants responsible of one or the other binding mode are not at all understood yet.

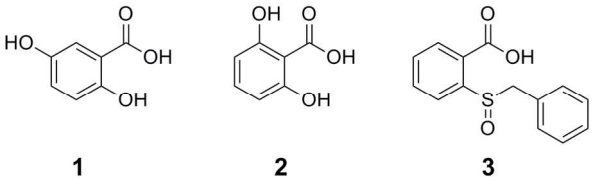
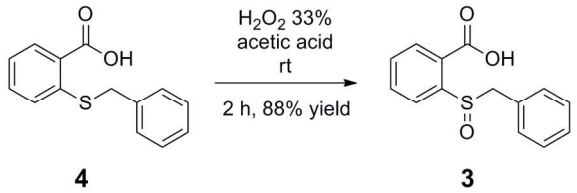


Figure 1. Chemical structures of compounds **1-3**

In a general research project aimed to investigate in more detail these compounds, we have undertaken a kinetic and structural study of an *ortho*-substituted benzoic acid, namely 2-benzylsulfinylbenzoic acid **3**, showing that this molecule not only possesses an interesting inhibition profile against several physiologically relevant hCA isoforms, but it also presents a binding mode completely different from any other class of CAIs investigated so far: this carboxylate binds in a pocket which is not situated within the enzyme active site, but **OUT** of it.

Compound **3** was synthesized by selectively oxidizing the sulfide **4** to the corresponding sulfoxide, as reported in the literature (Scheme 1).[30] Its physico-chemical characterization was in full agreement with the reported structure (see Supporting information).



Scheme 1. Synthesis of compound **3**

Sulfoxides have rarely been studied as ligands for transition metal ions in metalloenzymes, despite their zwitterionic form endowed with chelating properties. Furthermore, the sulfoxide moiety displays good synthetic versatility towards steric and electronic modifications, and induces a chiral center important in recognition by enzyme active sites. Thus, our initial working hypothesis was that the concurrent presence of two functional groups (COOH and S=O) could infer an ambidentate character to this derivative. The inhibition profile of four physiologically dominant isoforms hCAs I, II, IX and XII with compound **3** and acetazolamide (**AAZ**), as standard sulfonamide inhibitor, was investigated (Table 1) (see Supporting information for experimental details). These isoforms have been used in the assay due to the fact that the cytosolic hCAs I and II are widespread house-keeping enzymes, involved in a host of physiologic processes, whereas the transmembrane hCAs IX and XII are tumor-associated enzymes, abundant in hypoxic tumors and recently validated as antitumor targets.[1] Interestingly, hCA I was not significantly inhibited by benzoic acid **3** up until 10 μM concentration of inhibitor, whereas the other cytosolic isoform, hCA II, was inhibited in the submicromolar range (K_I of 0.15 μM). Moreover **3** was a micromolar inhibitor of hCA IX but did not significantly inhibit hCA XII. It is also interesting to note that the sulfide **4** did not significantly inhibit any of these CA isoforms (K_Is > 10 μM, data not shown). The inhibition data of **3** are rather atypical, prompting us to hypothesize a different mechanism of action with respect to all known inhibitors. For this reason we investigated in detail the interaction of the benzoic acid **3** with hCAs by means of X-ray crystallography, choosing hCA II for the study since it is easily to crystallize and the best studied isoform.[1]

Crystallization experiments on the unbound hCA II were performed, as previously described (see Supporting information for experimental details),[31,32] using the hanging-drop vapor-diffusion method and ammonium sulfate as precipitant. Crystals of the hCA II/**3** adduct were then obtained by soaking the crystals of the enzyme in a saturated solution of the inhibitor for 48 hours. The structure of the enzyme-inhibitor complex was analyzed by difference Fourier techniques using 1CA2 structure as starting model[33] and refined to a resolution of 1.50 Å with final crystallographic R-factor/R-free values of 0.163/0.184. Statistics for data collection and refinement are shown in Table S1.

Table 1. hCA I, II, IX and XII inhibition data with compound **3** and acetazolamide (**AAZ**, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide) as standard inhibitor, by CO_2 hydration, stopped-flow technique.

	K_I (μM)[a]			
Compound	hCA I	hCA II	hCA IX	hCA XII
3	>10	0.15	1.29	>10
AAZ	0.25	0.012	0.025	0.006

[a]Mean from 3 different assays, errors were within ± 5-10% of the reported values.

Analysis of the electron density maps within the catalytic site of the enzyme surprisingly did not show the binding of any inhibitor molecule, neither to the catalytic zinc ion nor to the residues in its immediate neighbourhood. On the contrary, an inhibitor molecule, rather well defined in the electron density (Figure 2), was observed in a cavity located on the protein surface, about 14 Å far from the Zn^{2+} ion, and delimitated by residues Gly6, Tyr7, Gly8, Asn11, His64, Phe231, Asn232 and Glu239 (Figure 3).

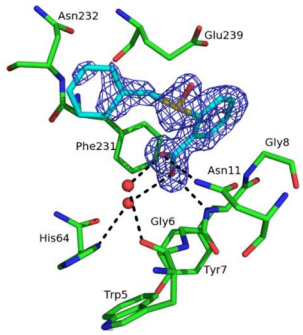


Figure 2. σA-weighted |2Fo–Fc| map (at 1.0 σ), relative to the inhibitor molecule **3**. Residues involved in inhibitor recognition are also shown.

The inhibitor was stabilized in this cavity by several polar interactions. In particular, one oxygen of the carboxylate moiety accepted a hydrogen bond from the backbone nitrogen atom of Tyr7, whereas the other oxygen interacted with the ND2 atom of Asn11 (Figure 2). Moreover, both oxygen atoms of the carboxylate group were involved in two strong hydrogen bonds with water molecules, which in turn were bound to the backbone oxygen of Trp5 and to the ND1 atom of His64. Interestingly, this latter water-mediated hydrogen bond froze the His64 residue in its *out*-conformation. Several other interactions stabilized the organic scaffold of the inhibitor within the cavity. In particular, the phenyl ring established a number of strong van der Waals contacts (distance < 4.0 Å) with residues Gly6, Tyr7, Gly8, Phe231 and Glu239, while the benzysulfinyl moiety interacted with Phe231 and Asn232 (Figure 2). Binding of inhibitors in this region of the enzyme has already been observed in the complexes which hCA II forms with two other benzoic acids, namely **1** and **2** (Figure 1). Indeed, the structural superposition of these latter two complexes with the one under

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investigation showed that the phenyl rings of the three inhibitors are perfectly superimposable and their interactions with water molecules and both enzyme residues Tyr7 and Asn11 are all retained (Figure 4).

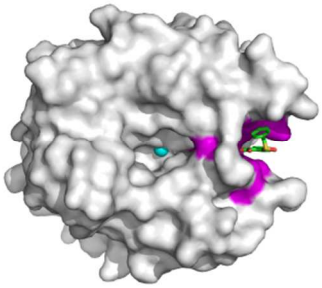

Figure 3. Solvent accessible surface of hCA II in its complex with **3**. The cavity where the inhibitor is bound is highlighted in magenta. The catalytic zinc ion is represented as a cyan sphere at the bottom of the active site cavity.

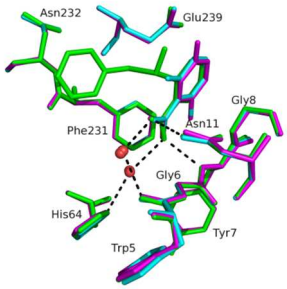

Figure 4. Structural superposition of the hCA II/**1** (magenta) (PDB code 4E3D), hCA II/**2** (cyan) (PDB code 4E3F) and hCA II/**3** (green) complexes in the protein region where compound **3** is bound.

However, it is worth noting that in hCA II/**1** and hCA II/**2** adducts another inhibitor molecule has been observed anchored to the zinc-bound water molecule.[29] In order to understand why **3**, although being a substituted benzoic acid like **1** and **2**, does not bind to the zinc-bound water molecule in the same manner of its analogues, we superimposed it to the molecules **1** and **2** bound in the active site. From this superposition it is clear that compound **3** in this position would strongly clash by means of its *ortho* substituent with residues which delimit the active site cavity (Figure 5).

It is important to highlight that this is the first time that the binding in the aforementioned cavity on the protein surface is not associated to a contemporary binding within the active site, thus indicating that it alone can cause the inhibition of the enzyme catalytic activity. Considering the catalytic mechanism, described above, which needs a flexible His64 to restore the active form of the enzyme, it is reasonable to hypothesize that this inhibition is due to the inability of His64 to change its conformation and thus participate in the proton shuttling needed to regenerate the nucleophilic species of the enzyme. Previously reported studies, showing that the mutation of His64 to Ala in hCA II caused a decrease in the rate of reaction by about 20 fold, are in agreement with this idea.[6] In these studies it was also shown that the decrease in catalysis could be rescued by the addition of exogenous proton acceptors in solution such as histamine, small imidazoles, pyridines and their derivatives.[6,34-37] Thus we decided to test our hypothesis verifying if the addition of imidazole could restore the enzyme catalytic activity (inhibited by **3**). The inhibition of hCA II by **3** with or without imidazole was then measured and the data are reported in Table 2. Analysis of these data shows a restoration of the proton shuttling activity blocked by **3**, even at low concentrations of imidazole (2 μM), with an almost total regain of the initial catalytic activity at 15-20 μM of imidazole in the assay system. It should be mentioned that imidazole itself acts as a CA activator (CAA),[38] binding in a site at the entrance of the active site cavity,[5] and providing an alternative route for proton release. We also tried to rescue the catalytic activity of hCA II complexed to **3** with a buffer which does not bind within the CA active site, such as HEPES or TRIS (in concentrations of 2-20 μM). In these cases no rescue of activity was observed, which is another confirmation of the proposed inhibition mechanism.

As mentioned above, compound **3** is a good hCA II and hCA IX inhibitor, but does not inhibit hCA I and hCA XII (Table 1). It is difficult to identify the reasons of this behaviour; however, an hypothesis can be done superimposing the residues defining the cavity where **3** is bound in hCA II, to the corresponding residues in the other isoforms (data not shown). From this superposition it can be observed that the substitution of Glu239 with an arginine in CA XII and of Gly8 with an aspartate in hCA I can cause a steric hyndrance for the positioning of the inhibitor. More generally, it can be seen that this cavity is delimited by different residues in the diverse isoforms, thus it can be considered an interesting target to be explored in drug design studies of selective CAIs.

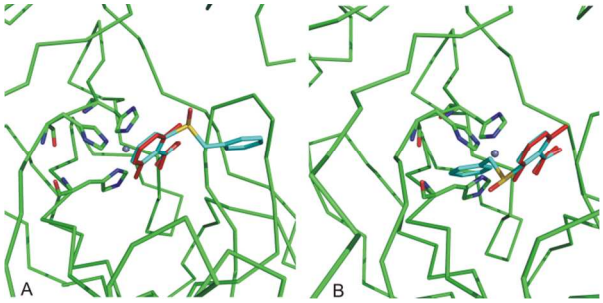

Figure 5. A detail of the active site of the hCA II/**1** adduct with inhibitor **3** superimposed to **1** in two different orientations (A) and (B), demonstrating that it is not possible its accommodation in the active site without clashing with enzyme active site residues.

Table 2. hCA II inhibition by 10 μM of benzoic acid **3**, varying concentrations of imidazole (Im), by a CO_2 hydration, stopped-flow technique.

Inhibitor	% CA activity[a]
No inhibitor	100%
3 10 μM	58%
3 10 μM + Im 2 μM	61%
3 10 μM + Im 5 μM	73%
3 10 μM + Im 10 μM	84%
3 10 μM + Im 15 μM	96%
3 10 μM + Im 20 μM	100%
Im[b] 20 μM	147%

[a]Errors in the range of 2-4 % from three different measurements; [b]Im alone is a known CA activator.

In conclusion, these findings, together with previously reported studies, indicate that carboxylic acids can present different inhibition mechanisms against CAs. Indeed, they can coordinate directly to the zinc ion, as observed for hCA II/formate[27] and hCA II/acetate,[28] form a hydrogen bond with the zinc-bound water molecule, as observed for hCA II/**1** and hCA II/**2**, and freeze the *out* conformation of His64, as observed for hCA II/**3**, even though it is difficult to predict *a priori* the binding mode adopted by a given carboxylate. Moreover, it cannot be excluded that the same molecule could

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adopt different binding modes to different CA isoforms. Altogether these data make carboxylic acids an interesting class of compounds in need of detailed investigations as inhibitors of metalloenzymes such as CAs.

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† Electronic Supplementary Information (ESI) available: Experimental details and Table of Crystallographic data. See DOI: 10.1039/c000000x/